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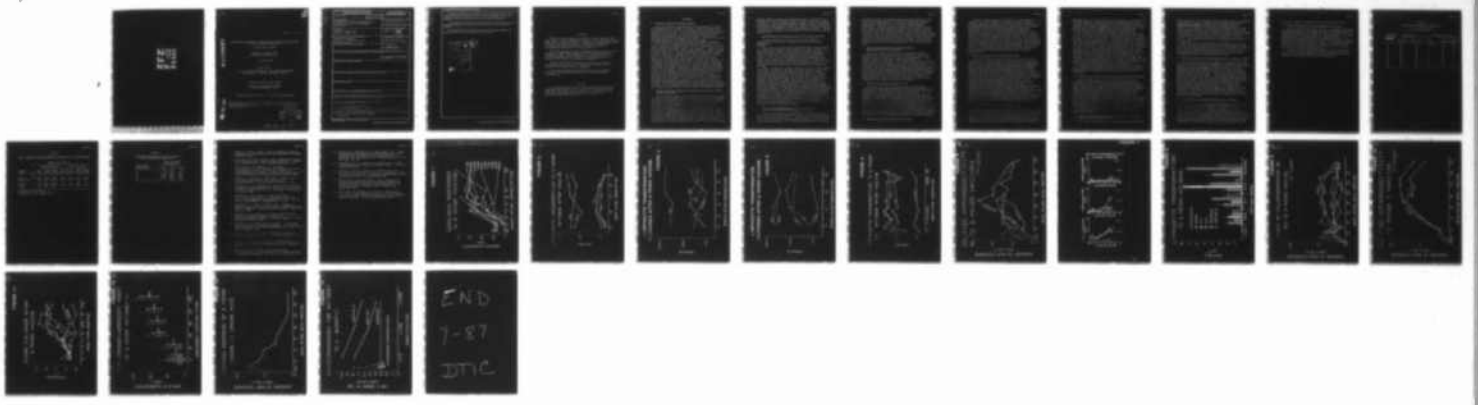

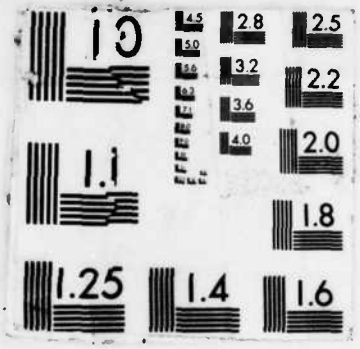
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Mechanisms of Protective Immunogenicity of Microbial Vaccines of Military Medical Significance

Annual Summary Report

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Monique A. Berman, Ph.D.

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Initial clinical studies of Phase I Q fever vaccine (IND #610) have have been undertaken this year in 18 volunteers at two dose levels. Local reactions to vaccine were minimal and without abscess or granuloma formation. Immunologic results show consistent antibody responses and variable lymphocyte transformation responses to Q fever antigens.

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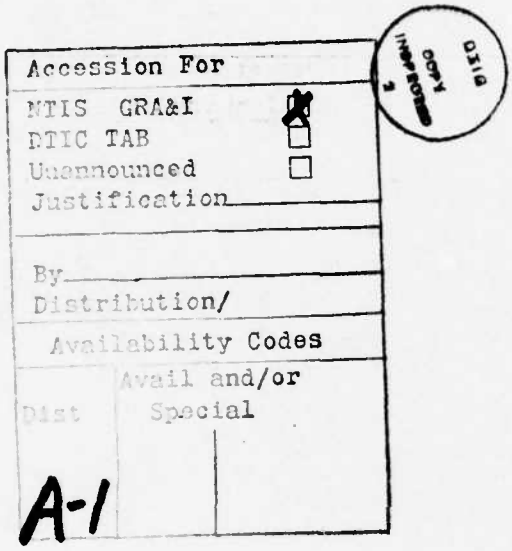
FIAX immunofluorescent assay procedures were used for serodiagnosis of Q fever and Legionnaire's disease and for detection of antigens from C. burnetii.

Hybridoma antibodies were prepared in mice to Q fever antigens and assayed by FIAX and a new radioimmunoassay technique.



SUMMARY

Initial clinical studies of Phase I Q fever vaccine (IND #610) have have been undertaken this year in 18 volunteers at two dose levels. Local reactions to vaccine were minimal and without abscess or granuloma formation. Immunologic results show consistent antibody responses and variable lymphocyte transformation responses to Q fever antigens.

Dermal granulomatous skin reactions seen to Q fever vaccine antigens in immune guinea pigs have been analysed for their immunologic basis. New information has been obtained on the interrelations between antigens of C. burnetii and their relative contributions to granuloma formation.

FIAX immunofluorescent assay procedures were used for serodiagnosis of Q fever and Legionnaire's disease and for detection of antigens from C. burnetii.

Hybridoma antibodies were prepared in mice to Q fever antigens and assayed by FIAX and a new radioimmunoassay technique.

Forward

In conducting the research described in this report, the investigator(s) adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Resources, National Academy of Science-National Research Council.

PROGRESS

1. Clinical Studies of Q Fever Vaccine

This year we have conducted the initial studies of the IND 610 Q fever vaccine in man. In brief, eighteen subjects have received vaccine to this point, twelve at the 6 ug dose level and six at the 30 ug level. No serious adverse reactions have been noted in these subjects. Local reactions have been limited to transient erythema and pain without evidence of abscess or granuloma formation as presented in Table 1. Immunologic results show the appearance of antibody measured by FIAX immunofluorescence in all subjects as illustrated in Figure 1.

The results of lymphocyte transformation (LT) testing of human volunteers are presented in Figures 2 through 5. In each case a mitogen and antigen control is included in addition to the specific Q fever antigens, phase I and phase II. Figure 2 shows an individual with no LT response (MFT) who had a brisk antibody response (Figure 1). Figure 3 shows another individual (MAB) with more baseline variation but no clear Q response. Figure 4 shows an individual (BRU) who clearly acquired high grade reactivity in response to immunization. Figure 5 shows the individual (MSA) with the most severe local vaccine reaction who evidenced pre-existing LT reactivity to both Q fever antigens. In response to immunization this individual showed a rapid and significant increase in reactivity. Further follow up of these individuals including 6 month skin tests and complement fixation titers, is in progress. Lymphocyte proliferative results prior to vaccine correlated with adverse local reactions in the two subjects with moderately severe reactions, but skin test responses were not impressive in these subjects. We conclude that the dose of antigen in the skin test may be inadequate to detect prior sensitivity to antigen in the vaccine and have proposed studies with larger doses of skin test in our contract renewal. The utility of lymphocyte testing in predicting vaccine reactions is not unexpected and will be pursued in more detail in our further studies based on these positive preliminary results.

2. Studies of Dermal Granulomatous Hypersensitivity in Q Fever Immune Guinea Pigs

Further studies this year on the granulomatous skin reactions seen to Q fever antigens in immune guinea pigs have concentrated on testing a variety of antigenic preparations of *C. burnetii* for their relative ability to induce or elicit such reactions. The findings of the studies are summarized in the attached abstract. In more detail, the skin reactions were all qualitatively similar when either Phase I vaccine, Phase II vaccine, or TCA soluble and insoluble extracts were used to skin test Phase I immune animals as illustrated in Figure 6. When animals were immunized with the various preparations and skin tested with whole cell Phase I vaccine, it was the group of Phase II immune animals which showed accelerated granuloma formation at three weeks as illustrated in Figure 7. Parallel lymphocyte transformation studies revealed a high degree of cross reactivity

between Phase I vaccine and TCA residue with a very selective response of Phase II immune animals to Phase II vaccine as shown in Figure 8. These findings together suggest that the metabolic alteration which results in phase variation to Phase II produces preferential expression of a specific "Phase II antigen" which is a significant contributor to the granulomatous reaction. Further studies are in progress in this model with other purified antigen preparations.

3. Immunofluorescent Studies of Q Fever Skin Test Reactions

Immunofluorescence has been a useful technique for demonstration of microbial antigens in cells, including C. burnetii (1).

Method:

Skin test sites and their surrounding tissue were excised from immune and nonimmune guinea pigs and frozen sections (7 um) were prepared on slides. The slides were briefly fixed in acetone. Sections were overlaid with guinea pig anti-phase I Q fever vaccine serum. Normal guinea pig serum was used as a control. After a 10 min incubation at room temperature, the slides were rinsed with PBS and overlaid with FITC-labelled rabbit anti-guinea pig Ig (Behring Diagnostics). After a 15 min incubation, the sections were rinsed in PBS, mounted in 90 % glycerol-10 % Tris buffer pH 9, and examined by UV microscopy.

Results:

Phase I immune and nonimmune control guinea pigs were skin tested with a 1:10 dose of phase I Q fever vaccine. Skin test areas were examined by immunofluorescence using guinea pig anti-phase I serum on frozen sections from day 1, 3, 7, and 9 after intradermal injection. The results show that Q fever antigens are retained in the skin of nonimmune animals on day 1 and day 3 after skin testing. The pattern of fluorescence indicates sparse distribution of antigen in the dermis. In contrast, no antigen was detectable in the skin test areas of immune guinea pigs at day 1 and day 3, suggesting that antigen had been cleared or processed and was no longer reactive with our antiserum to phase I. In future experiments these sections will be analyzed with our mouse hybridoma antibodies which should be very sensitive probes for various antigenic determinants of C. burnetii. In addition, lymph nodes have been collected and will be examined for the presence of antigen. In late (day 9) reactions from immune guinea pigs, isolated small groups of cells with fluorescent cytoplasm were found. This cytoplasmic fluorescence appeared granular and may represent phagocytised antigen or antigen-antibody complexes.

4. Effect of Cyclophosphamide Pretreatment on Dermal Granulomatous Hypersensitivity to C. burnetii

A useful technique for assessing the immunologic basis of skin reactivity has been the use of high dose cyclophosphamide (CY) prior to immunization (2). This drug has selective toxicity for B cell function in the guinea pig, leaving T cell activity unchanged or enhanced. In our previous study with CY on guinea

pig DTH to Q fever we demonstrated enhanced DTH and protection after such CY pretreatment even though antibody formation to vaccine was suppressed (3). We have now applied the same experimental procedure to the analysis of granulomatous sensitivity. Guinea pigs were injected with 250 mg/kg CY and three days later were immunized with 12 ug of Q fever vaccine emulsified in either Freund's complete or incomplete adjuvant. Ten days later, the animals were skin tested in the flank by our usual procedure. As illustrated in Figure 9, the CY pretreatment resulted in enhanced granulomatous reactivity as measured by late induration in the presence of either adjuvant. This finding leads us to believe, as has been shown in other systems, that this granulomatous form of hypersensitivity is T-cell mediated (4,5).

5. Failure of Serum Transfer of Granulomatous Hypersensitivity to C. burnetii

One of the easiest and cleanest immunologic tests for determining the basis of skin reactivity is the transfer of immune serum. Such tests are used in standard Arthus assays, passive cutaneous anaphylaxis assays and atopic or Prausnitz-Küstner reactions. As an important control in the Q fever system, we transferred 2 ml of 3 wk immune guinea pig serum into groups of normal animals followed 1 hr later by skin testing. There was absolutely no significant reactivity apparent in either transfer group compared to controls (data not shown). Since serum did not transfer granulomatous hypersensitivity to Q fever vaccine, further experiments will include cell transfers from immune donors. These experiments will be done in a mouse model which will allow us to study the effect of lymphoid subpopulations (B, Fc^+, T helper, T suppressor cells) on the development of granulomatous hypersensitivity.

6. Bioassay of Q Fever Skin Test Antigen

Our skin test results in humans showed minimal skin reactivity in immunized volunteers at 8 weeks and 6 months after immunization. One possible cause of this result was that the rebottled skin test antigen prepared separately by Merrill-National Drug from a vial of IND 610 vaccine had lost potency in preparation or was unstable at such high (1:382) dilution. To test that possibility, a vial of Skin Test Antigen was compared with a fresh 1:382 dilution of lot 5 vaccine in guinea pig skin test and lymphocyte transformation (LT) assays. As illustrated in Figure 10, the similarity of these two preparations in skin test reactivity is striking. The LT results are not shown here but show a similar correspondence between the two products. There is nothing remarkable about these findings other than showing the utility of these cellular immune bioassays in determining antigenic potency of vaccines. These findings will be pursued in other antigenic systems in our next proposal.

7. Experimental Fever After Q Fever Vaccine

Bacterial vaccines have a potent source of reactogenicity in proportion to their content of endotoxin. Such toxicity, although clinically undesirable, has immunologic significance in providing adjuvant activity in some vaccines (6). C. burnetii has endotoxic activity measured by several standard test procedures (7,8,9,10) as well as toxicity in endotoxin non-responder mice (11). These observations prompted us to assess the potential for the IND 610 Q fever vaccine to cause fever in normal guinea pigs. Animals were held for two hours until staple rectal temperatures were obtained. Two groups of animals were then injected with 60 ug of vaccine, one IV and one IP and one group served as a control. As can be seen in Figure 11, the injection of vaccine produced prompt and high grade fever with the characteristics of an endotoxin response. All animals survived this experiment with no obvious adverse consequences. This assay will provide a useful method for assessing the relative toxicity of various purified Q fever antigenic preparations.

8. Lymphocyte Proliferation Testing of Sheep

One very promising natural experiment which we have been considering as a future test for vaccine efficacy is the fact that sheep in southern California carry and shed C. burnetii in high frequency. We are part of a consortium of investigators from UC Davis, UCSF, UCLA and other institutions organized to study the epidemiology and immunology of Q fever in sheep. Although this work is generally outside the scope of, and not sponsored by, this contract, the ability of Q fever vaccine to induce immunity in sheep will be reported through this mechanism as results develop. To that end we have conducted a pilot study on the ability to measure lymphocyte proliferate responses to mitogens and Q fever antigens in sheep. After several unsuccessful attempts at lymphocyte separation by standard sedimentation and centrifugal procedures including Ficoll-Hypaque, we were able to detect high level proliferative responses in sheep cells with the whole blood method, first introduced by us in rickettsial systems in guinea pigs (12). The data from such an early experiment comparing whole blood cultures with Ficoll-Hypaque separation are presented in Table 2. Although the sheep in question was seronegative for evidence of Q fever infection, the slight response of lymphocytes to Q fever antigens is consistent with a viable assay technique. These findings will be pursued in detail under other auspices.

9. FIAX Immunofluorescence Testing for Q Fever

In the last two years our laboratory has performed biosurveillance testing on over 1000 samples from 23 institutions inside and outside of California using our previously described FIAX technique (13). Since our previous report we have broadened our pool of complement fixation (CF) positive specimens several fold and present the additional data in Figure 12. The large numbers of positive samples clearly illustrate the sensitivity of FIAX testing and its reliability for screening serum samples.

Although there is little increase in fluorescence over the 16-128 CF range, there is a dramatic jump in fluorescence between CF titers of 8 and 16, the critical point of discrimination between "positive" and "negative" samples. Parallel testing of 40 of our samples by the unit at USAMRID revealed agreement between their micro IF test and our FIAX results in 38 of 40 specimens. Further exchange of diagnostic specimens is in progress.

As a result of the recent laboratory outbreaks (14,15), two features of Q fever are bound to receive more attention in the future, the chronic disease state and endocarditis. The hallmark of these states is the presence of high titer specific antibody to phase I C. burnetii antigen. Given the fact that we have over 100 random positive sera on hand, we elected to survey a group of them for the presence of phase I antibody by FIAX. The thought was that a high phase I FIAX value may provide an early predictor of those at risk for chronic manifestations of Q fever. The results of this testing revealed a straightforward correlation between the two tests with no evidence of high phase I titers in this pilot group. The highest Phase I FIAX value was in a vaccinee. Two other samples with positive phase II FIAX from suspect chronic cases were negative for phase I antibody. At the time of our forthcoming vaccine trial at UCSF, selected follow up sera from their cases of 1979 will be studied for antibody to both antigens in an attempt to test our hypothesis.

We have also applied the FIAX procedure to the detection of Q fever antibody in mice as illustrated in Table 3. This assay will provide a rapid method for indirect testing for infectious rickettsiae in our ongoing studies of latent and chronic infection.

10. FIAX Detection of C. burnetii Antigens in Biologic Specimens

One attractive feature of the FIAX immunofluorescence procedure is its ability to detect antigen or antibody in a quantitative manner. We have conducted several experiments on the relative merit of various direct and sandwich techniques to detect antigen and conclude that the direct coating of antigen on sticks followed by conjugated antibody is as good as any other procedure in detecting antigen down to the 80 ng/ml level. Increasing the affinity of the paper carrier for antigen by precoating with specific antibody has not increased the sensitivity of the test. The direct approach will now be used to test specimens from the naturally infected sheep described above. The relevance of these techniques to vaccine development is in the area of potency testing for vaccines, whereby a candidate preparation could be rapidly and quantitatively assayed by this FIAX technique. This approach is in progress with different Q fever antigen preparations and is the basis for work with inactivated Venezuelan encephalitis virus vaccine presented in the renewal proposal.

11. Delayed Hypersensitivity and FIAX Serologic Testing with Legionnaire's Disease Bacterium

Based on our prior experience with delayed hypersensitivity

(DTH) testing in the tularemia bacterial model (16), we attempted to characterize skin reactions to inactivated preparations of Legionella pneumophila in guinea pigs immunized with the Legionnaire's disease (LD) bacteria in Freund's complete adjuvant. The results to date have been disappointing in that direct (endo)-toxicity is a prominent feature of the skin test reactions in normal pigs and no specific increase is noted in the immune animals. The results of these experiments show absolutely no evidence of specific DTH to the LD bacterium in immune animals. We conclude, in contrast to a previous report of DTH to LD (17), that this technique is not a promising one in this disease model.

Similarly, the FIAX procedure for serodiagnosis of LD was performed in immune guinea pigs and in a limited manner in man. There was a good correlation between FIAX and standard IF testing in a few high titer positive specimens tested but due to the complicated species variation now known to be present and to the low military relevance of the LD problem at present, these investigations will not be pursued at present.

12. Assay for Granulomatous Hypersensitivity to Q Fever in Mice

Current knowledge of murine lymphoid cell populations would allow the study of the immune mechanism of granuloma formation in Q fever. We have therefore attempted to reproduce granulomatous hypersensitivity to C. burnetii in Balb/c mice. Mice were immunized with Q fever phase I vaccine in complete Freund's adjuvant and challenged 3 weeks later with a 1:100 dilution of vaccine (25 ul) in the footpad. Footpad swelling was measured with a "Schnelltaster" caliper. The results (Figure 13) show that swelling is optimal at 24 hr and gradually declines over a period of 10 days. This time course of induration differs from the guinea pig skin test which shows maximal induration around day 9 and does not therefore appear to represent a typical granulomatous hypersensitivity reaction. In subsequent experiments, the skin tests were done on the shaved flanks of mice and measured with a caliper adapted to measure skin fold thickness. This method induced reproducible induration to specific antigen in a group of tularemia immune mice. Current experiments are designed to determine suitable doses of Q fever vaccine in this assay.

13. Production of Hybridoma Antibodies to C. burnetii

Balb/c mice were immunized and boosted twice with phase I Q fever vaccine. Three days after the last booster immunization, immune spleen cells were fused with a nonsecreting P_3 myeloma cell line using polyethylene glycol. Hybrids were grown in selective HAT medium. Over 200 growing clones were screened by radioimmune assay (see below) for the production of anti-phase I and anti-phase II C. burnetii antibodies. Six clones producing antibodies to C. burnetii vaccine were selected for recloning and growing in vitro. The antibodies from these supernatants will be concentrated and studied further for their specificity. These monoclonal antibodies will be used in our immunofluorescence

studies of lymphoid organs and dermal granulomas in Q fever.

14. Radioimmunoassay for Mouse Antibody to C. burnetii

A plate binding RIA was adapted for the testing of hybridoma antibodies to phase I and phase II vaccine. Briefly, vaccine at various dilutions (neat to 1:100) in 50 ul was allowed to bind to Falcon 3911 Micro Test III plates at room temperature for 30 min. Unbound material was washed off with distilled water. Supernatants containing hybridoma antibodies were then added to the plates and allowed to bind during a 1 h incubation. Unbound antibody was removed by washing with PBS. Radiolabelled (125-I) rabbit IgG anti-mouse Ig was then added for 1 h, and unbound material was carefully aspirated. The bottom of each well was cut out and counted in a gamma counter.

Figure 14 shows binding and titration of two mouse sera (anti-phase I and anti-phase II) on plate-bound phase I and II vaccine. Individual points on the graph represent values for undiluted supernatants from various hybrid clones.

TABLE 1

CLINICAL AND IMMUNOLOGIC RESULTS OF

Q FEVER IMMUNIZATION

Experimental subject	Skin test result (mm at 24 hr)		Vaccine reaction		
			Pain	Erythema	DURATION
	Erythema	Induration	(onset)	(cm diameter)	(days)
1	22	0	+ (4 hr)	12	8
2	8	0	"	3	3
3	10	0	"	4	3
4	0	0	"	0	-
5	0	0	"	0	-
6	0	0	"	0	-
7	0	0	"	0	-
8	0	0	"	0	-
9	0	0	"	0	-
10	0	0	"	0	-
11	0	0	"	0	-
12	0	0	"	0	-

Table 2

SHEEP LYMPHOCYTE TRANSFORMATION TO MITOGENS AND Q FEVER ANTIGENS

	Additive to culture						
		Phytohemagglutinin		Q fever vaccines			
Culture method	None	LPS (lug)	PHA-2 (1:100)	PHA-3 (1:1000)	QI-1 (1:10)	QI-2 (1:100)	QII-1 (1:10)	QII-2 (1:100)
Whole blood(1:20)	67*	300 (4.5)+	667 (10.0)	1088 (16.2)	169 (2.5)	396 (5.9)	179 (2.7)	300 (4.5)
Ficoll-Hypaque	61	62 (1.0)	1181 (19.4)	1016 (16.7)	72 (1.2)	71 (1.2)	140 (2.3)	75 (1.2)

*CPM/Well (6 replicates)
+Stimulation index (Exp/control)

Table 3

FIAX Immunofluorescence Assay of Phase II Q Fever Antibody in Mouse Sera

Weeks after immunization	FIAX value(FSU) Serum dilution		
	1:10	1:40	1:80
0	8.4	11.4	3.6
2	7.1	11.4	6.1
4	96.5	79.6	39.4
8	143.0	87.0	37.7
14	-	65.2	43.8

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FIGURE 1

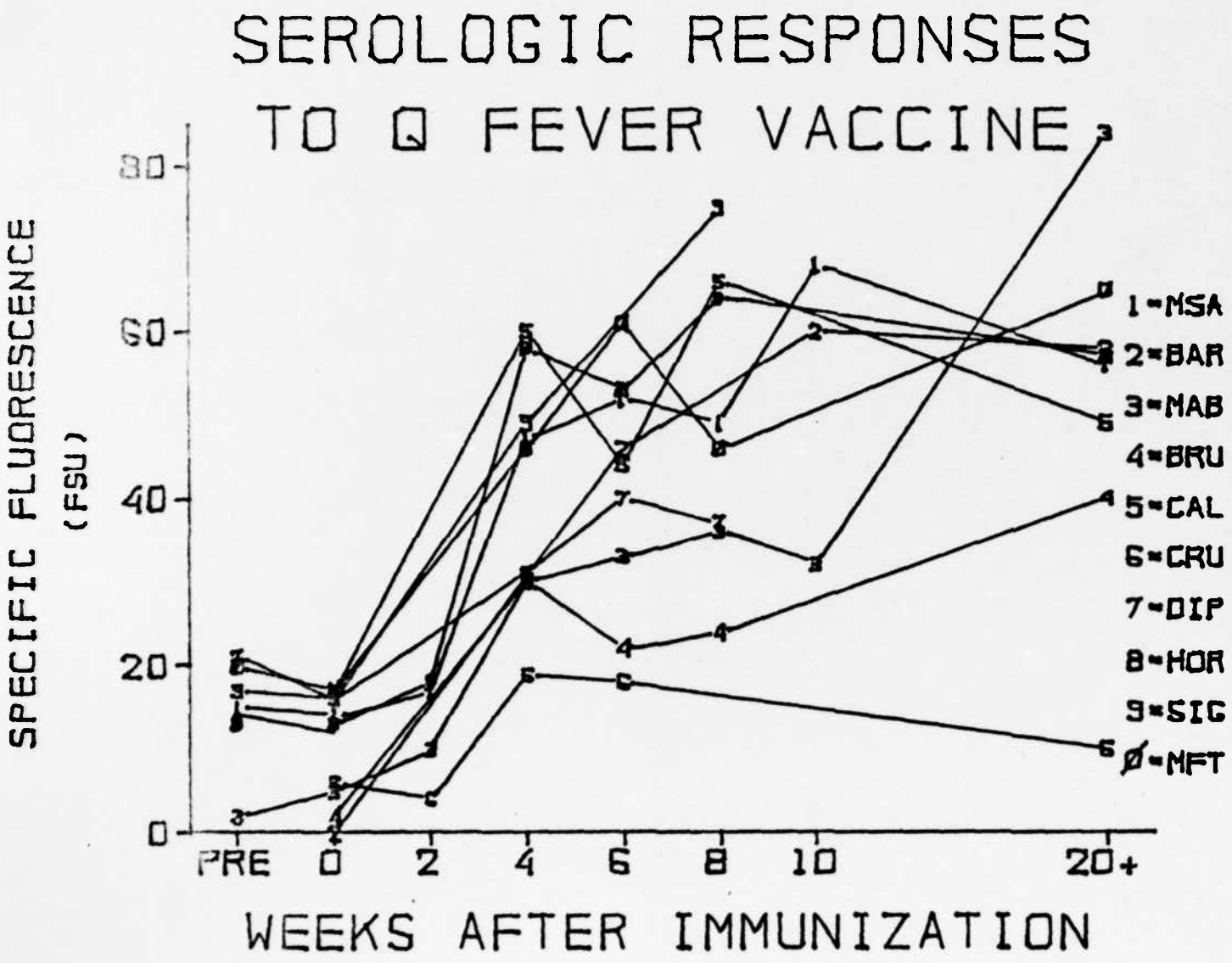
SEROLOGIC RESPONSES
TO Q FEVER VACCINE
SPECIFIC FLUORESCENCE
(FSU)
80
60
40
20
0
PRE
0
2
4
6
8
10
20+
WEEKS AFTER IMMUNIZATION
1=MSA
2=BAR
3=MAB
4=BRU
5=CAL
6=CRU
7=DIP
8=HOR
9=SIG
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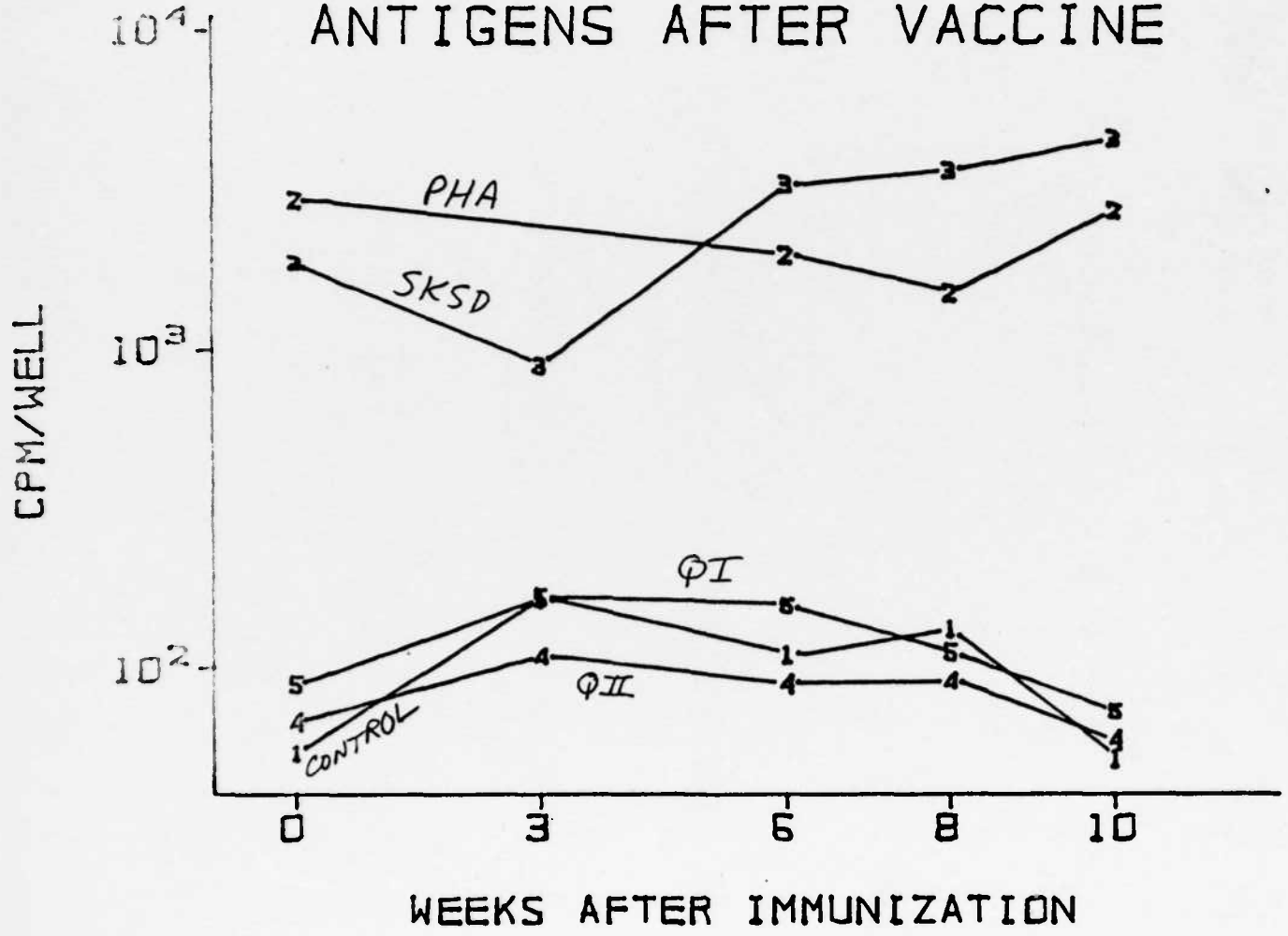
FIGURE 2



LYMPHOCYTE TRANSFORMATION RESPONSES AFTER Q FEVER VACCINE

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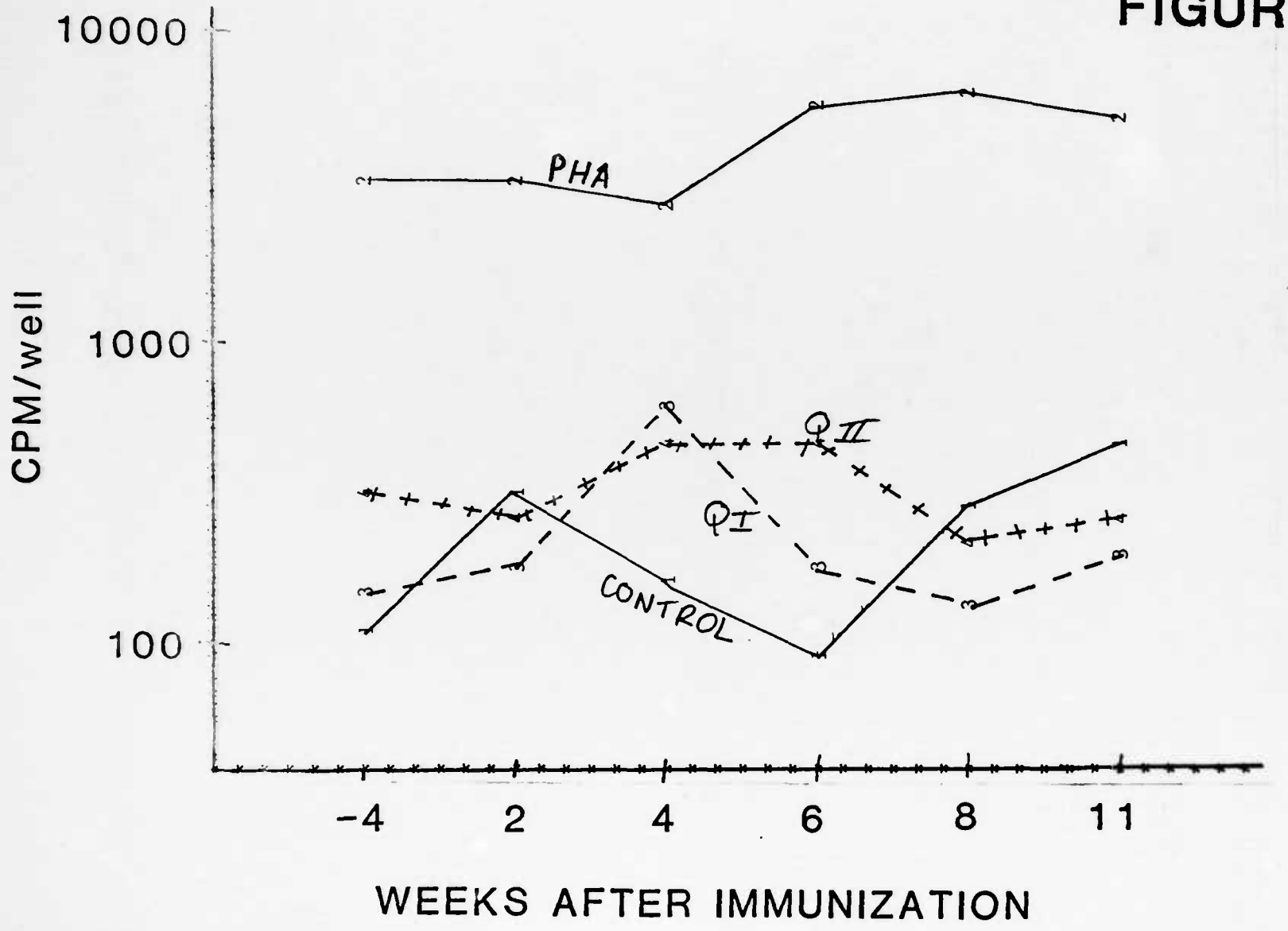
FIGURE 3



LYMPHOCYTE TRANSFORMATION RESPONSES AFTER Q FEVER VACCINE

FIGURE 4

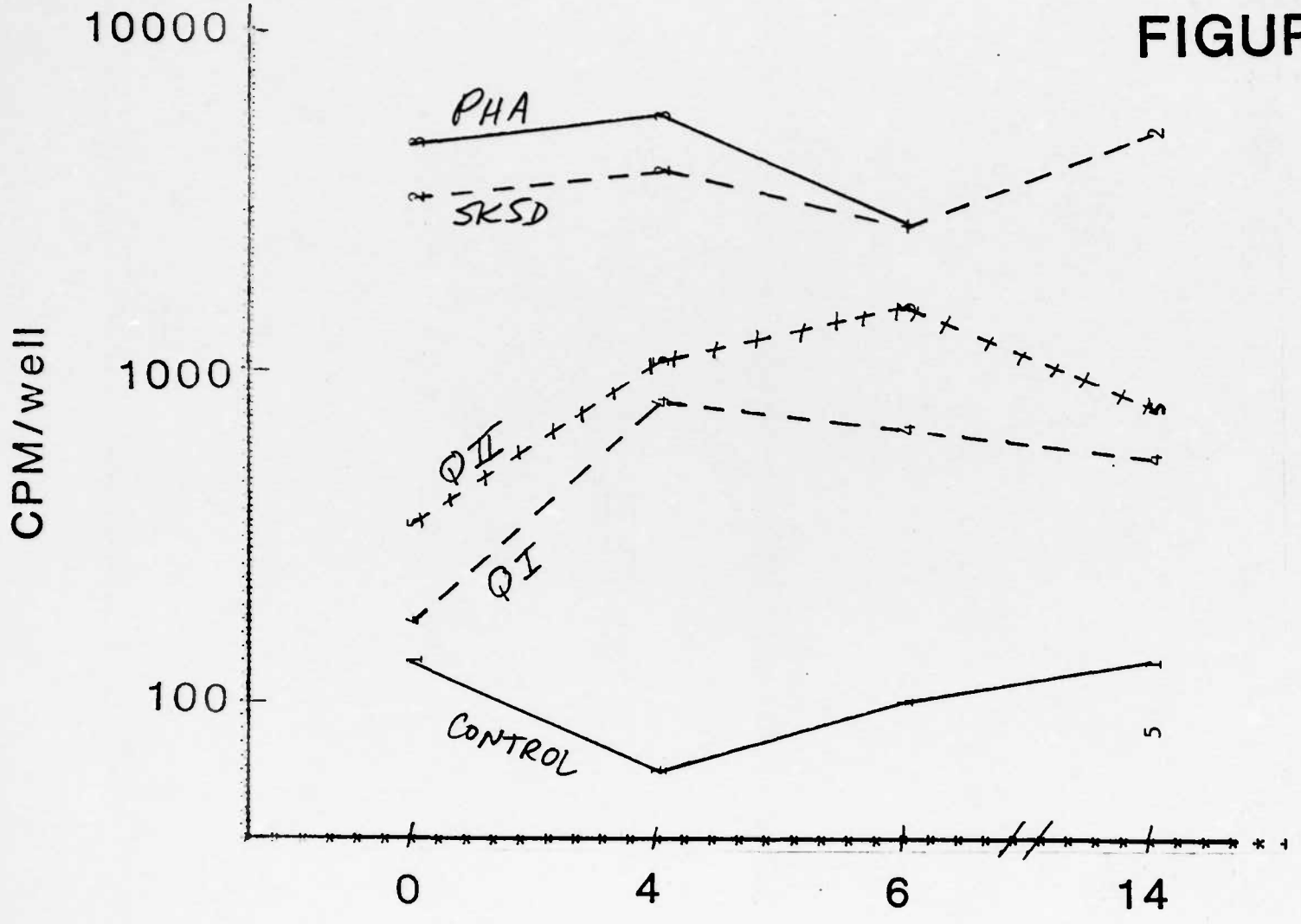


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FIGURE 5

LYMPHOCYTE TRANSFORMATION TO Q FEVER ANTIGENS AFTER VACCINE

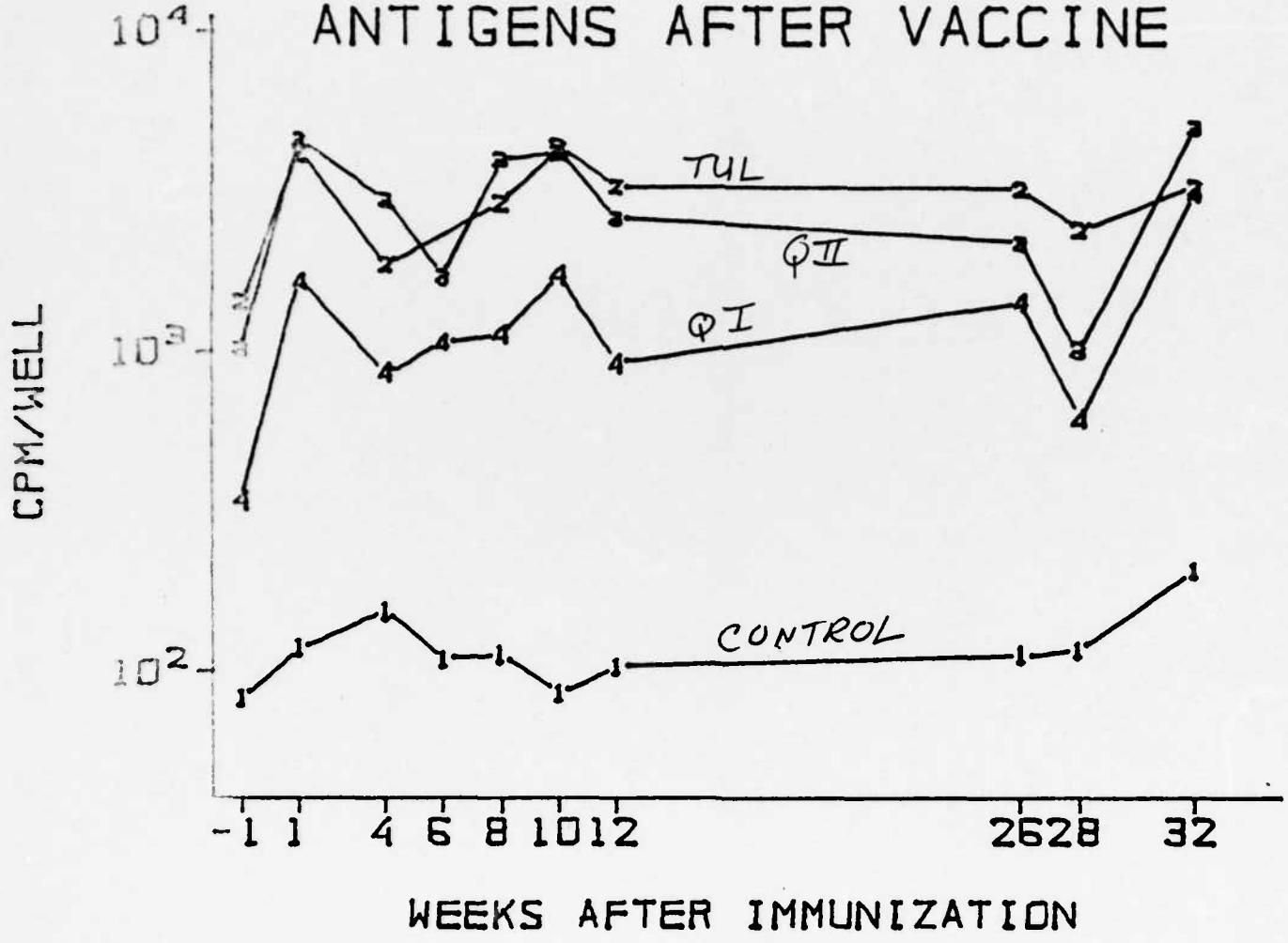


FIGURE 6

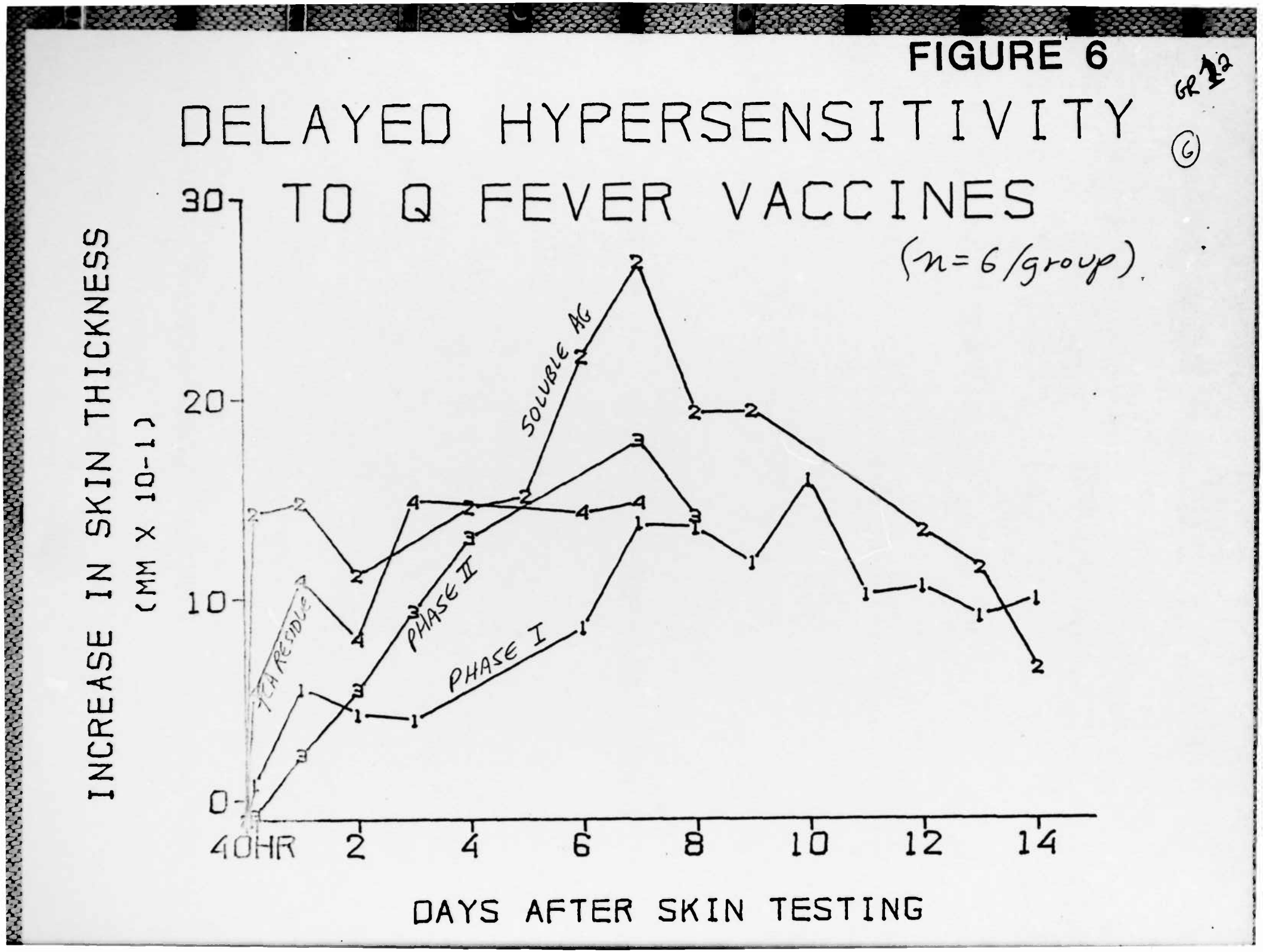

DELAYED HYPERSENSITIVITY
TO Q FEVER VACCINES
(n=6/group)
INCREASE IN SKIN THICKNESS
(MM X 10-1)
30
20
10
0
40HR
2
4
6
8
10
12
14
DAYS AFTER SKIN TESTING
SOLUBLE AG
PHASE II
PHASE I
7CA RESIDUE

FIGURE 7

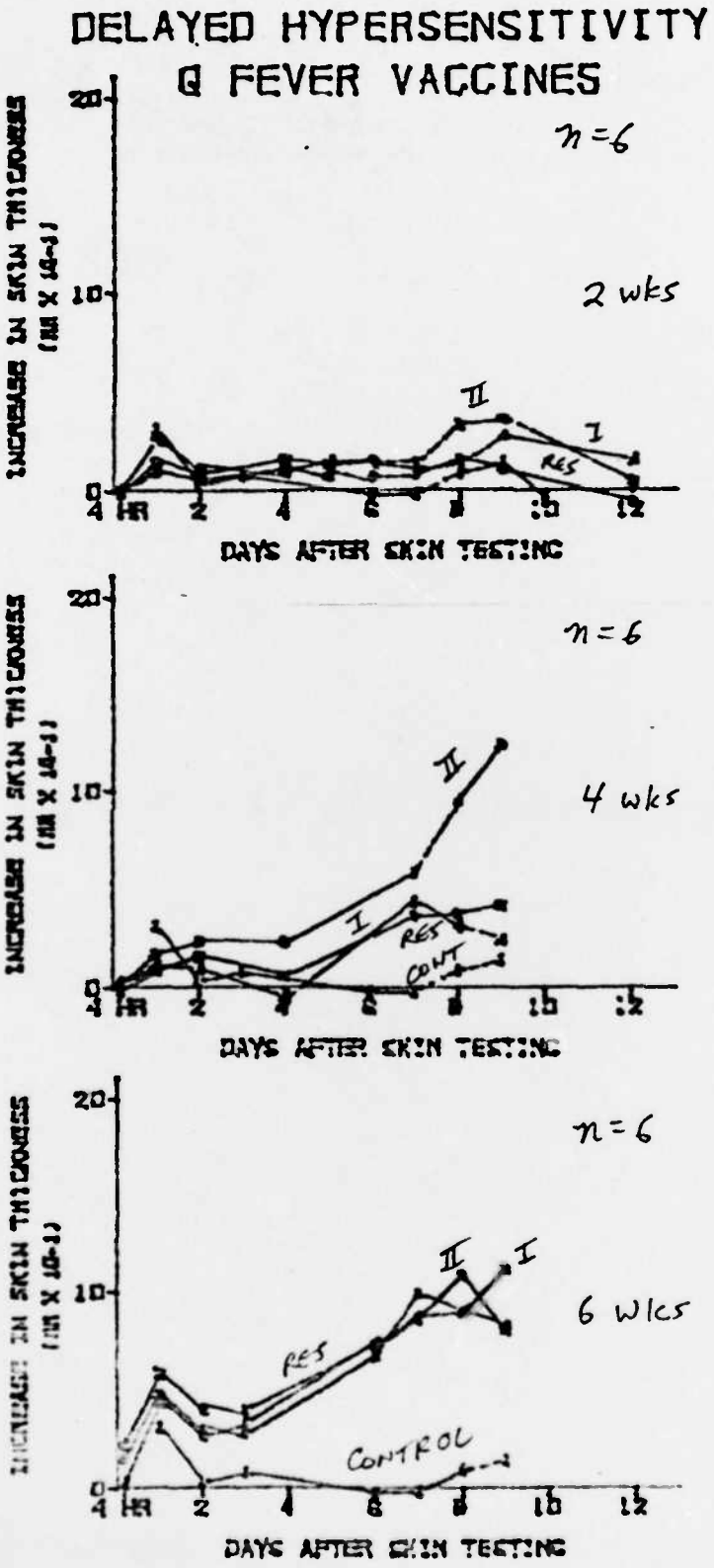

DELAYED HYPERSENSITIVITY
Q FEVER VACCINES
n=6
2 wks
II
I
RES
20
10
0
4 HR
2
4
6
8
10
12
DAYS AFTER SKIN TESTING
n=6
4 wks
II
I
RES
CONT
n=6
6 wks
II
I
RES
CONTROL

FIGURE 8

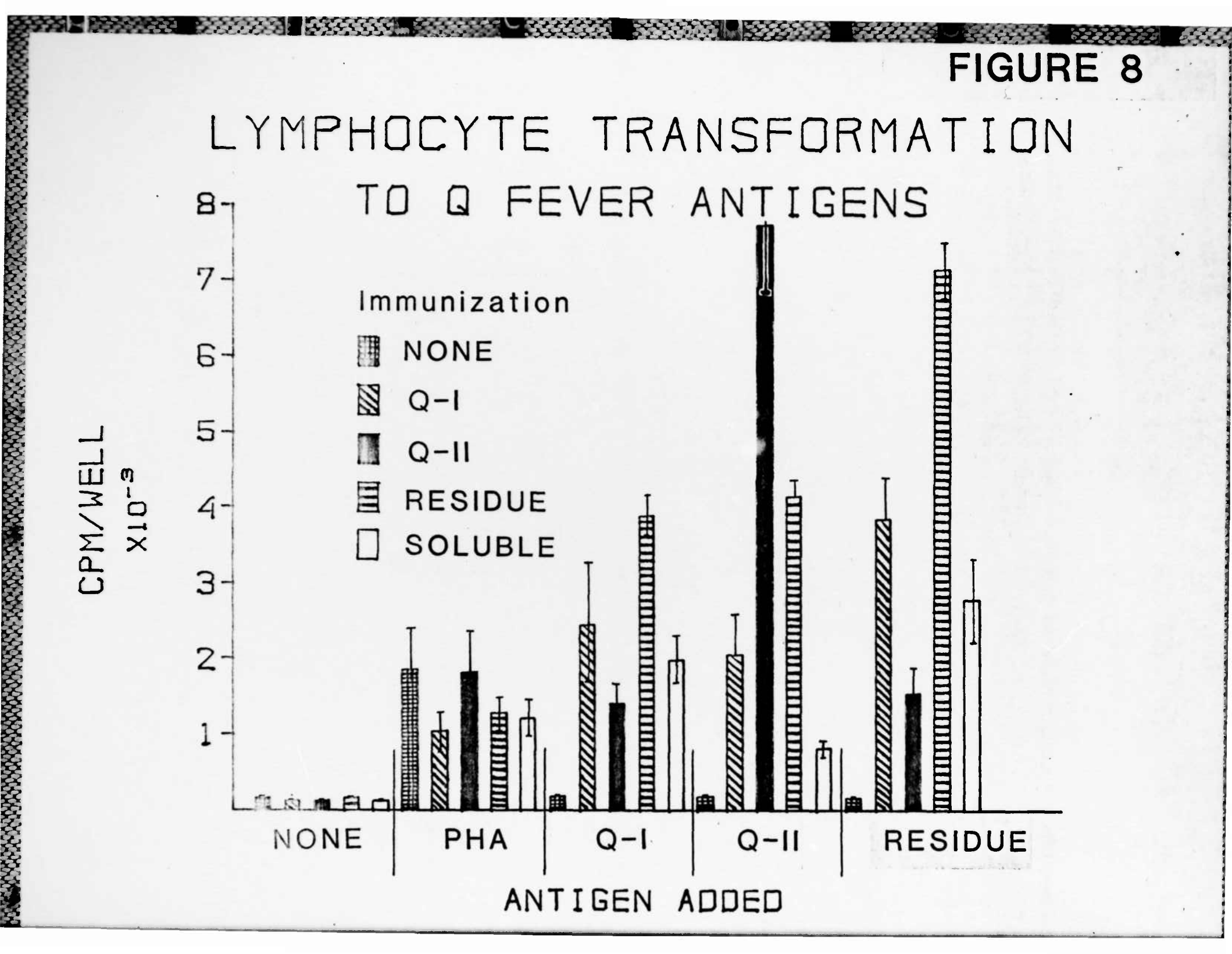

LYMPHOCYTE TRANSFORMATION
TO Q FEVER ANTIGENS
CPM/WELL
X10-3
8
7
6
5
4
3
2
1
Immunization
NONE
Q-I
Q-II
RESIDUE
SOLUBLE
NONE
PHA
Q-I
Q-II
RESIDUE
ANTIGEN ADDED

FIGURE 9

GR14
(9)

EFFECT OF CYCLOPHOSPHAMIDE ON DTH TO Q FEVER VACCINE

(n = 6/group)

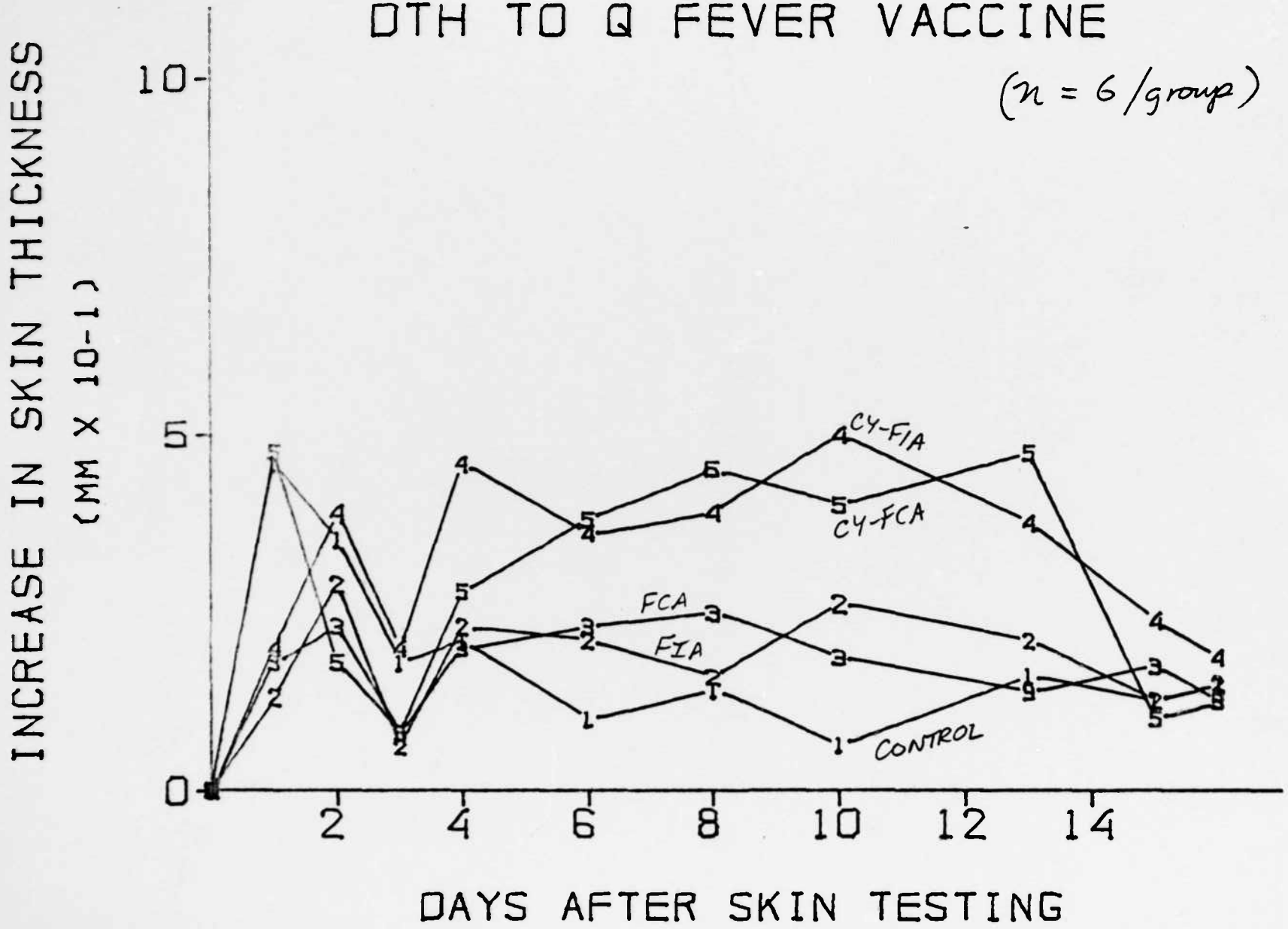
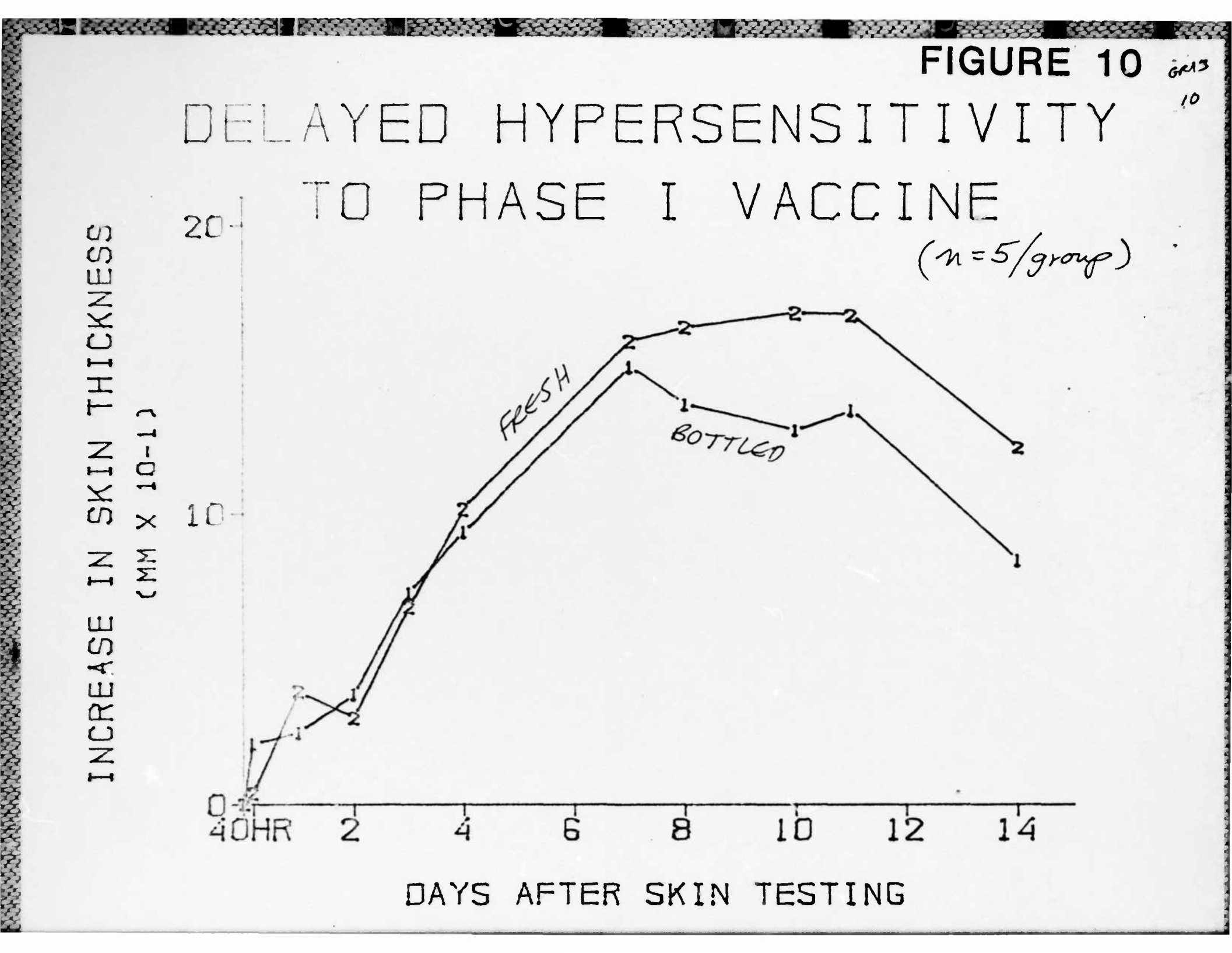


FIGURE 10


DELAYED HYPERSENSITIVITY
TO PHASE I VACCINE
(n=5/group)
INCREASE IN SKIN THICKNESS
(MM X 10-1)
20
10
0
FRESH
BOTTLED
40HR
2
4
6
8
10
12
14
DAYS AFTER SKIN TESTING

GA(17)

FIGURE 11

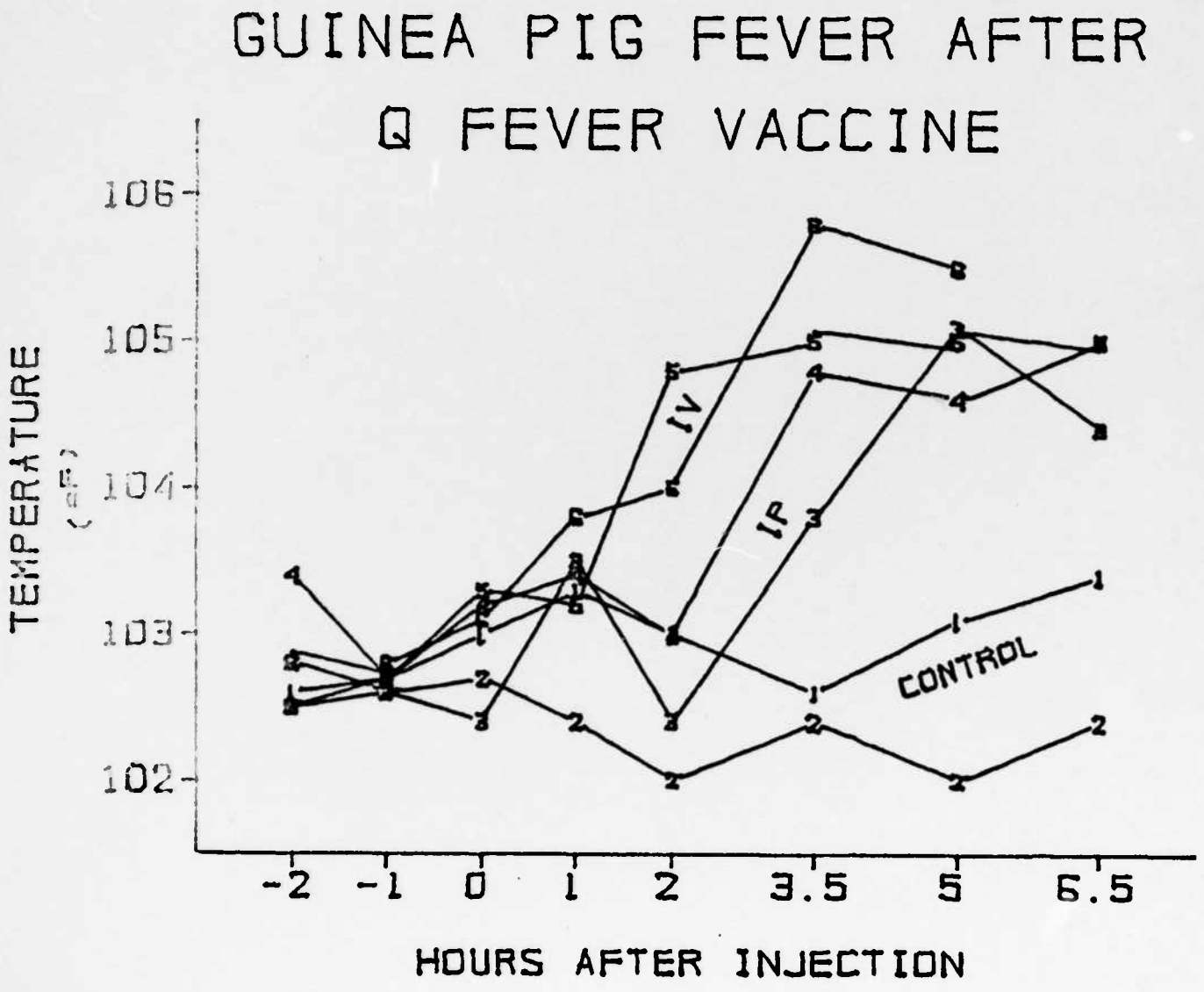


FIGURE 12 (12)

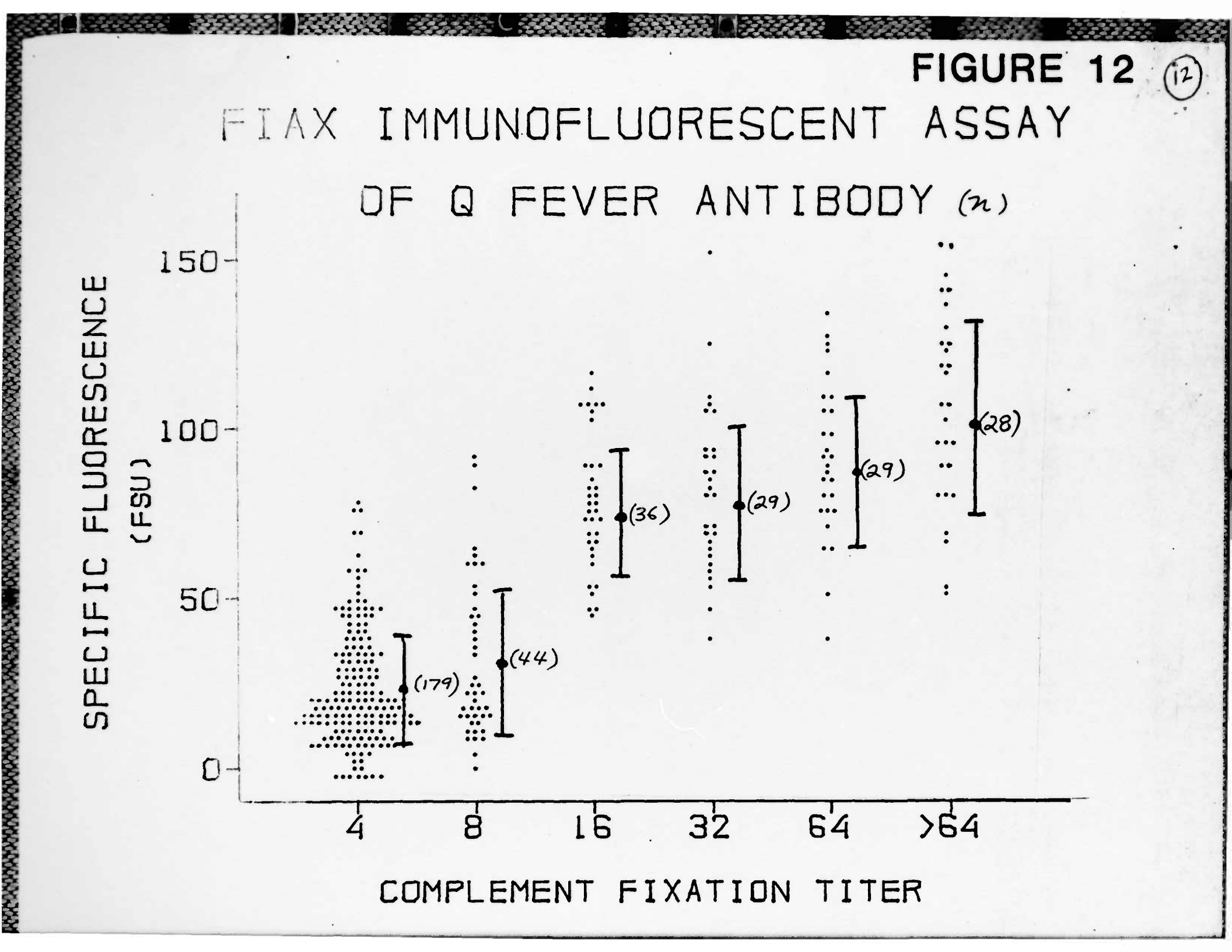

FIAX IMMUNOFLUORESCENT ASSAY
OF Q FEVER ANTIBODY (n)
SPECIFIC FLUORESCENCE (FSU)
150
100
50
0
(179)
(44)
(36)
(29)
(29)
(28)
4
8
16
32
64
>64
COMPLEMENT FIXATION TITER

FIGURE 13

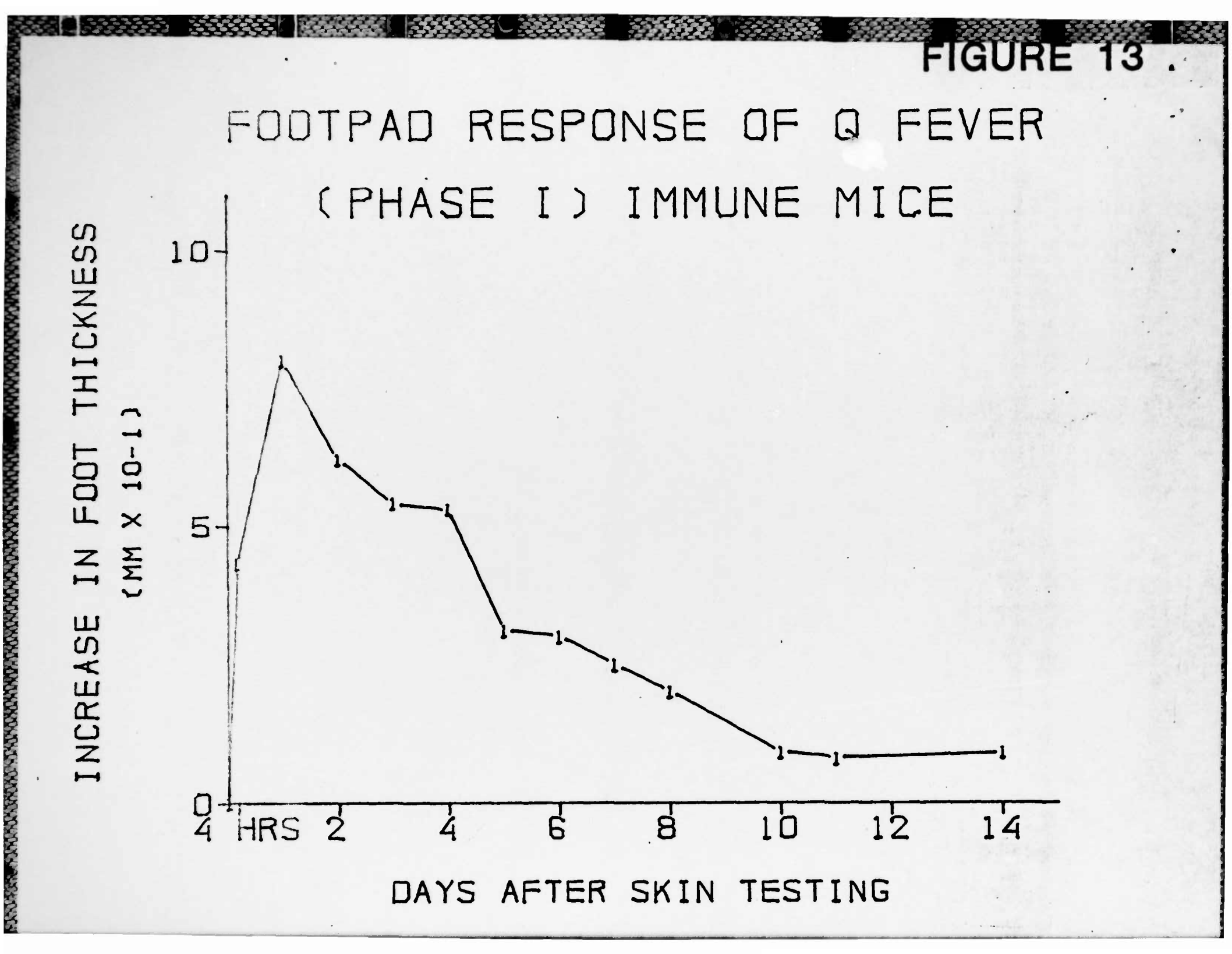
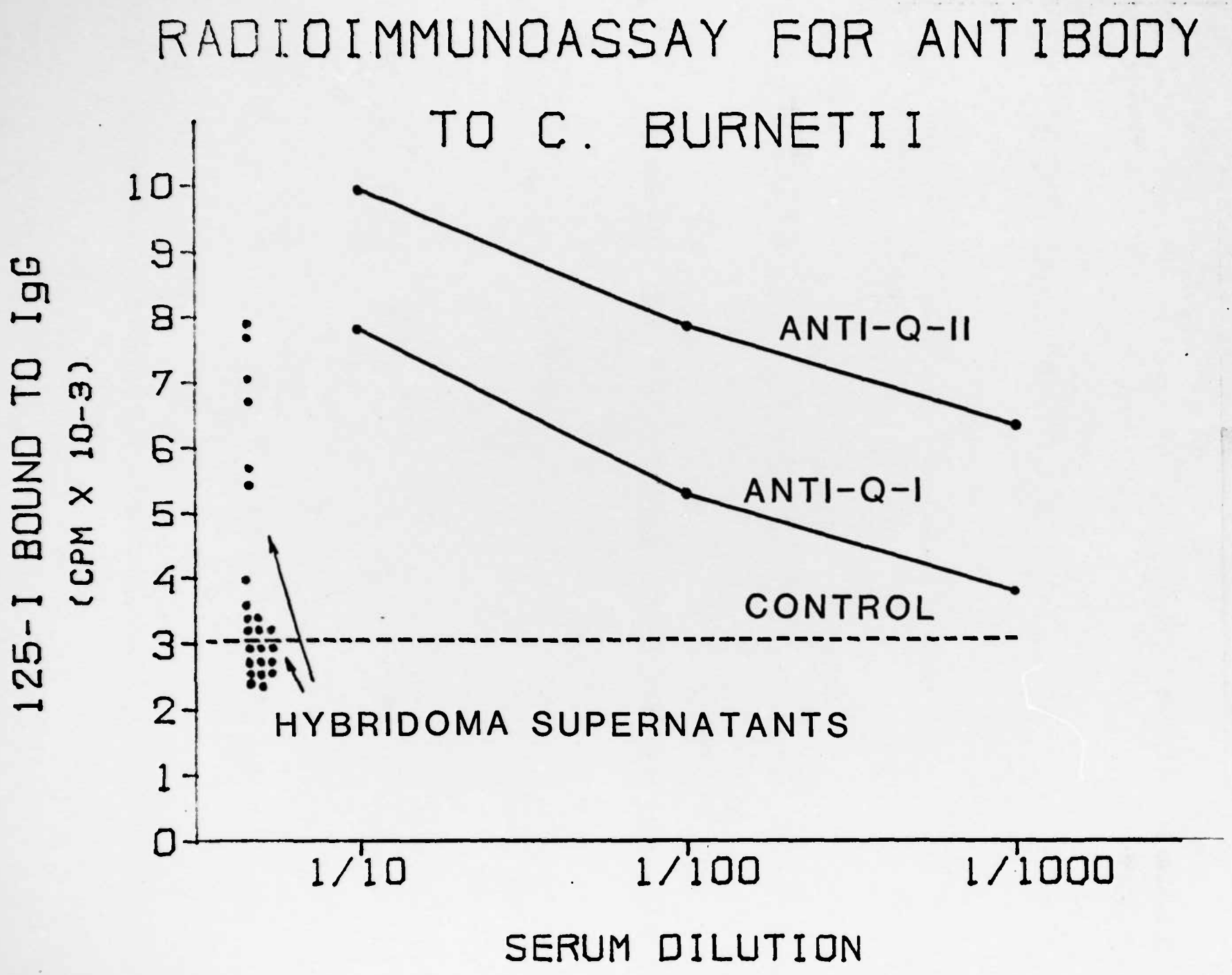

FOOTPAD RESPONSE OF Q FEVER
(PHASE I) IMMUNE MICE
INCREASE IN FOOT THICKNESS
(MM X 10-1)
10
5
0
4 HRS
2
4
6
8
10
12
14
DAYS AFTER SKIN TESTING

FIGURE 14


RADIOIMMUNOASSAY FOR ANTIBODY
TO C. BURNETII
125-I BOUND TO IgG
(CPM X 10-3)
10
9
8
7
6
5
4
3
2
1
0
ANTI-Q-II
ANTI-Q-I
CONTROL
HYBRIDOMA SUPERNATANTS
1/10
1/100
1/1000
SERUM DILUTION

END

7-87

DTIC